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MOLECULAR CHARACTERIZATION OF TOXIGENIC AND ANTIBIOTIC RESISTANT OF *STAPHYLOCOCCUS AUREUS* OF RECURRENT BOVINE MASTITIS

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ABSTRACT

To enhance the diagnosis of *Staphylococcus aureus* mastitis and its prospective antibiotic resistance in dairy cattle, a multiplex polymerase chain reaction (PCR) assay was developed for concurrent species identifycation, detection antibiotic resistant phenotypically and genotypically to penicillin (*blaZ gene*), gentamycin (*aac(6')* *aph* (*2'')genes*), and tetracycline (*tetK*, gene) and recognize the toxogenic patterns of *S. aureus* in cow's milk with recurrent clinical mastitis. Bacterial culturing was carried out on 179 bovine recurrent clinical mastitic milk samples resulted in 84 (46.9%) *coagulase positive staph* isolates, while 69 (38.5%) confirmed by molecular identification by PCR as *S. aureus*. Also some virulence factors as enterotoxin A and B (Sea and *Seb*) genes were determined in 7(10.1%) and 1 (1.4%) respectively in confirmed isolates. As well as determined drugs resistance and their genes. The results of antimicrobial susceptibility tests showed 100%. 43.5% and 58% resistance for penicillin, gentamycin and tetracycline respectively. While genotypically were 100%, 40.6% and 53.6% for (*blaZ*, *aac* (*6'*) *aph* (*2''*) *and tetK*, respectively. This study reports the presence of multidrug resistant *S. aureus* in recurrent clinical mastitis with highly virulent toxic genes that could be a major obstacle in the treatment and control of mastitis in dairy farms causing highly economic impacts and recommended that the polymerase chain reaction (PCR)-based assays to detect pathogens associated with mastitis and that it has several advantages, including rapid results and high sensitivity.

Key words: *Mastitis, drug resistance, staph. aureus*

INTRODUCTION

In developing countries like Egypt, bovine mastitis consider a major problem in dairy farms and farmers incur heavy economic losses due to expenditures for treatment and reduced milk production Mastitis is a multi-etiological disease, and appropriate control is based on knowledge of the etiology, thus identification of pathogens is a fundamental aspect of mastitis control programs. whatever, *Staphylococcal aureus* is the most predominant major contagious bacteria studied in dairy farms as they critical source of subclinical and clinical intra-mammary infections rely on the epidemiological studies and mastitis control efforts. Leading to a major problem with zoonotic implications and severe financial losses worldwide (Ebtsam *et al.*, 2014; Pamela, 2014).

The pathogenic potential of *S. aureus* based on numerous cell surface virulence factors and their capability of producing a variety of exotoxins and cell surface-associated proteins that enhance the cellular attachment, invasion to host immune system and stimulation of toxic tissue reactions.

(Kalorey *et al.*, 2007; Hussain *et al.*, 2012b). The most identified toxins gene were (*sea* and *seb)* from *S. aureus* isolates from the mastitic milk (Mousa *et al.*, 2017).

Improper use of antimicrobials has resulted in augmenting the bacterial resistance mechanisms hinder the treatment and control of *S. aureus* mastitis as well as intra-mammary infusion for preventive measures in dry cows therapy (David and Daum, 2010).

S. aureus strains are capable of mutation, clonal evolution and horizontal gene transfer that boost up the virulence and drug resistance (Brody *et al.*, 2008). Hence, identification of pathogenic and resistant *S. aureus* from intra mammary infection at herd level is of vital importance for successful treatment and control. Generally changes in mastitis isolate profiles influenced by setting have been reported earlier which again emphasizes the need for periodic evaluation of

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S. aureus in terms of virulence and antibiotics resistances. Currently, penicillin, gentamycin, erythromycin, and tetracycline are frequently used for the treatment and control of mastitis. Many studies have recommended polymerase chain reaction (PCR)-based assays to detect pathogens associated with mastitis and that it has several advantages, including rapid results and high sensitivity.

Therefore, the accurate and rapid diagnosis of genetic variability specially antibiotic resistance genes is necessary to identify the genetic relatedness of strains and their source of spread; and one of the preferred reliable and broad genotyping methodologies is repetitive element by PCR (Gandhale *et al.*, 2017).

Our aim is to investigate the genotypic distribution, enterotoxigenic virulence and resistance patterns of *S. aureus* strains isolated from mastitic cattle in some Egyptian dairy farms.

MATERIALS AND METHODS

Bacterial Strains

A total of 179 milk samples from recurrent clinical mastitic cows from three dairy farms were collected with briefly history of various animal husbandry practices. After sanitizing, 10ml of Milk was collected in sterile vials, samples were transported at 4˚C and the milk samples were plated onto blood agar, nutrient agar, Baird-Parker agar supplemented with egg-yolk tellurite emulsion and Manitol salt agar (oxid UK) at 37 ºC / for 48 hrs. All isolates were confirmed as S. aureus by Gram staining, catalase activity, tube coagulase test In addition to colony morphology and type of hemolysis produced, and then suspected colonies were extra identified by catalase test and tube Coagulase.

In udder health and neonates Department Laboratory Animal Reproduction Research institute (ARRI) Egypt, the bacteriological culture and biochemical identification for isolates were done according to National Mastitis Council, (1999).

Antimicrobial susceptibility tests:

Antimicrobial susceptibility was tested by Kirby–Bauer disk diffusion method and the minimal inhibition concentrations (MICs) of the antibiotics on Muller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (2014). The investigated antibiotics disks were from (Oxid UK), penicillin G (P, 10 IU), gentamicin (CN, 10 µg), and tetracycline (TE, 30 µg). Pure cultures of *S. aureus* were grown in brain–heart infusion (BHI) broth and incubated at 37 °C for 18 h. BHI broth cultures were further evenly spread on MHA (Oxid UK) plates. Then inoculated antimicrobial disks were left then at room temperature for 30 min followed by incubation at 37 °C for 24 h to measure the inhibition zone diameters. Strains were classified as resistant, intermediate, or susceptible on the basis of the size of the inhibition zone (in millimeters) and MICs used for interpretation the diameters of the zones of inhibition were as published by Clinical and Laboratory Standards Institute (2014).

Table (1)

Antibiotics	zones of inhibition (in millimeters)		
	Susceptible	Intermediate	Resistance
Penicillin G(10IU)	≥29	21-28	≤20
Tetracycline (30 µg)	≥19	15-18	≤14
Gentamycin(10 µg)	≥15	13-14	≤12

DNA Extraction and Detection of Selected Resistance Genes by PCR

Molecular identified of *S. aureus* and molecular characterization of some virulence and resistance genes as (*Sea*, *Seb*, *blaZ*, *tetK* and *aac* (*6'*) *aph* (*2''*)), started with extraction of *S. aureus* DNA from overnight-grown at 35°C S. aureus cultures in Brain Heart Infusion (BHI) Broth (oxide UK) according to QIAamp DNA mini kit instructions (Catalogue no.51304), then each DNA sample go in PCR with mixing it with PCR mixture according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit. (PCR Master Mix ((Takara) Code No. RR310A), PCR grade water.

Oligonucleotide primers with specific sequence gene (Metabion (Germany)) for each virulence character and amplify a specific product as shown in Table (2).

Table (2): Oligonucleotide primers sequences

Gene	Sequence	Amplified product	Reference
16-23s rRNA	Forward: TTCGTACCAGCCAGAGGTGGA Reverse: TCTTCAGCGCATCACCAATGCC	228 bp	**Pradhan *et al.* (2011)**
Sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102 bp	**Mehrotra *et al.* (2000)**
Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164 bp	
blaZ	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC	173 bp	**Duran *et al.* (2012)**
aac(6')aph (2'')	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491 bp	
tetK	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360 bp	

And cycling conditions of the primers during cPCR, temperature and time conditions of the two primers during PCR are shown in Table (3) according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit.

Table (3): Cycling conditions of the different primers during cPCR:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16-23s rRNA	95ºC/45s	95ºC/45s	1 cycle	95ºC/45s	35 cycles	1 cycle
Sea, Seb,	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec	72˚C 30 sec	35	72˚C 7 min.
blaZ, tetK, aac(6') aph (2'')	94˚C 5 min.	94˚C 30 sec.	54˚C 45 sec	72˚C 45 sec	35	72˚C 10 min.

The PCR product visualized through running on agarose 1.5% gel electrophoresis containing 0.5 mg ethidium bromide in 0.5× Tris-EDTA electrophoresis buffer) at 100 V and photographed under UV illumination. (Sambrook *et al.,* 1989) with modification and using DNA Molecular weight marker. The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded, the power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet, the gel was photographed by a gel documentation system and the data was analyzed through computer software.

DISCUSSION

Staphylococcus aureus is the most common etiological pathogen of bovine mastitis possessive many virulent factors and multidrug resistant make the disease difficult to cure increasing global problem which has become a high concern for dairy industry worldwide. So determine the antimicrobial susceptibility profiles is required not only for effective therapy but also for monitoring the spread of resistant strains in defined ecological niches (Hogan and Smith 2003; Coelho *et al.,* 2009). Present study showed that the antimicrobial susceptibility profiles of S. aureus

were determined and high levels of resistance to penicillin followed by tetracycline and then gentamycin were detected.

The study carried out on 179 recurrent clinically mastitic dairy cows in 3 dairy farms. The bacteriological examination revealed that 8.4% showed no bacterial growth, this result was lower than that recorded by Ebtsam (2001) who recorded that 17.28% of the milk samples had no bacterial growth, also (Ashraf *et al.,* 2017) who reported that no bacterial growth of 30% of collected samples, that indicated the need for another specific pathogen media or spontaneous cure or intermittent shedding of M.O.. While 91.6% of samples showed bacterial growth, out of them 84/179 (46.9%) coagulase *Staphylococci* (*CPS*) isolates and 80/179 (44.7%) other isolates**.**

We assayed the genotypes of 84 *Coagulase positive Staph* strains isolated from recurrent clinical bovine mastitic milk samples which revealed 69 (38.50%) isolates identified as *S. aureus* strains by PCR.

Present results agree with that previously reported by Bedane *et al.* (2012) who mentioned that approximately 30%-40% of all mastitis cases caused by *S. aureus* and nearly to that reported by Mousa *et al.* (2017) who found the prevalence rate of *S. aureus* from mastitis was 26.7%. On the other hand, higher prevalence 75% recorded by Jørgensen *et al.* (2005).

Mastitic milk can possess a serious hazard to human consumers due to higher bacterial count or toxins. *S. auerus* enterotoxins (SE), particular SEA-SEE were the most classical discovered genes in cattle, the isolates showing 8/69 (11.6%) *S. aureus enterotoxogenic type* A and B gens by PCR as 7/69 (10.1%) and 1/69 (1.5%) *were Sea and Seb* genes respectively. This result was lower than that was recorded by Yu-Cheng *et al.* (2008) as they found *sea* (29.2%) and seb (19.7%), from *isolated S. aureus* strains and Mousa *et al.* (2017) recorded enterotoxin *sea* and *seb* genes in *S. aureus* isolates from subclinical mastitis milk were the most prevalent with 60% and (50%), respectively using multiplex PCR.

S. aureus antimicrobial resistant strains were determined by disc diffusion assay and the corresponding resistance genes were determined by PCR. The results showed penicillin; gentamycin and tetracycline resistance results were 100%, 43.5% and 58%respectively. These results were different to that recorded by Yang *et al.* (2016) as antimicrobial resistances of *S. aureus* were (84.09%, 9.09% and 15.91%) for penicillin, gentamycin and tetracycline respectively.

Acquisition of resistance in *S. aureus* isolates attributed to mutation in gene or due to exchange of genetic material between organisms, since resistance genes carrying mobile genetic elements of *S. aureus* have exceedingly been explored (Teruyo *et al.,* 2003). The molecular characterization by PCR determination of drug resistance genes (*blaZ*) for penicillin), genes (*aac* (*6'*)*aph* (*2''*) for aminoglycoside antibiotic as (gentamycin) and gene (*tetK*) for antibiotic tetracycline were 100%, 40.6% and 53.6 % respectively.

The *bla*Z gene detected in all isolates, these findings are consistent with previous report (Haveri *et al.,* 2007) and agree with the results of Flayhart *et al.* (2005) who reported (97.6%) agreement between different molecular and culture methods. Tetracycline resistance encoding gene tetK was present in 53% of isolates which is lower than previously detected in 96% isolates (Gao *et al.,* 2011).

Mechanisms of resistance to antibacterials are so complex that the presence or absence of a certain resistance gene does no certainly indicate that the particular isolate is resistant or sensitive to the corresponding antimicrobial agent (Gow *et al.,* 2008).

RESULTS

Table (4): Bacteriological results of milk samples collected from recurrent clinical mastitis cows.

FARMS	recurrent clinical cases	Culturing of milk samples									
		Coagulase positive Staph (CPS)		*CPS* with other M.O		All *CPS*		other M.O		Neg. Bact.	
	No.	No.	%	No.	%	No.	%	No.	%	No.	%
Farm 1	**46**	**12**	**26.1%**	**12**	**26.1%**	**24**	**52.2%**	**17**	**37.0%**	**5**	**10.8%**
Farm 2	**55**	**16**	**29.1%**	**10**	**18.2%**	**26**	**47.3%**	**25**	**45.5%**	**4**	**7.2%**
Farm 3	**78**	**18**	**23.1%**	**16**	**20.5%**	**34**	**43.6%**	**38**	**48.7%**	**6**	**7.7%**
Total	**179**	**46**	**25.7%**	**38**	**21.2%**	**84**	**46.9%**	**80**	**44.7%**	**15**	**8.4%**

All *coagulase positive isolates* were confirmed as *S. aureus isolates* by PCR using 16s–23s ISR rRNA genes which resulted in 69 (38.5%) isolates of *S. aureus.*

Antibiotic resistance profile of *S. aureus*

In this study all number of the isolates were resistant to penicillin G 69/69(100%), some were resistant to tetracycline (58%). less resistance was observed in gentamycin (43.3%), (Table5)

Table (5): Drug Resistance

Antibiotics	Resistance		Moderate		Sensitive	
	No	%	No	%	No	%
Penicillin G	69	100	0	0	0	0
Gentamycin	30	43.5	3	4.3	36	52.2
Tetracycline	40	58	6	8.7	23	33.3

Table (6): Relationship between the phenotypic and genotypic antibiotics resistance to penicillin, tetracycline, erythromycin and gentamicin in *S. aureus*

	S. aureus (N=69)			
Gene	molecular characterization (PCR)		Phenotypically (sensitivity test)	
	No.	%	No.	%
(Penicillin***) blaZ***	69	100	69	100
(Gentamycin)aac(6')aph (2'')	28	40.6	30	43.4
(Tetracycline***)tetK***	37	53.6	40	58

Table (7): *S. aureus* enterotoxin detection by PCR (N=69).

Gene	***S. aureus*** enterotoxin Identification	
	No.	%
enterotoxogenic type A (*Sea)*	**7**	**10.1**
enterotoxogenic type B (*Seb)*	**1**	**1.4**

N =(69) equal the total molecular identified of ***S. aureus*** isolates

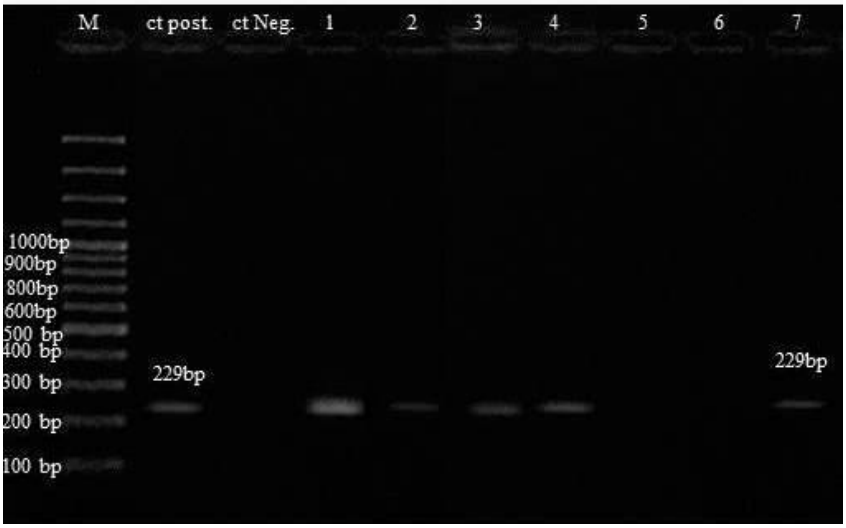

Figure (1): Electrophoresis gel show results of PCR amplification of 16s–23s ISR rRNA gene for detection of *S. aureus* gene, Lane M: 100 bp DNA ladder, Lane ct. pot.; control positive, Lane ct. Neg.; negative control; Lanes 1-4 and 7: PCR amplified 229 bp product of *S. aureus* Positive, Lane 5 and 6 are negative.

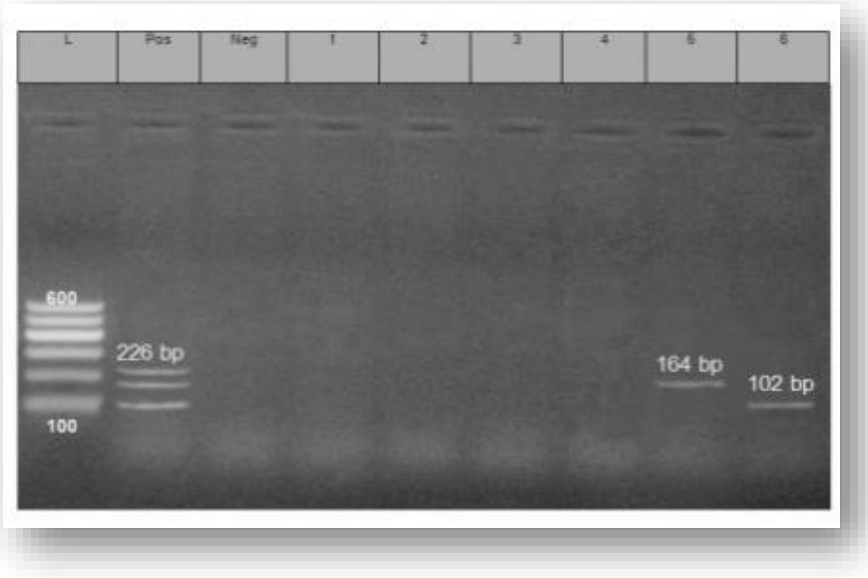

Figure (2): Agarose gel electrophoresis of products on a 2% agarose gel from multiplex PCR of *sea*, *seb* gene, Lane M: 100 bp DNA ladder, Lane (Pos.); positive control; Lane (N); negative controls; Lanes (5) PCR amplified 164 bp product of *seb* gene, Lanes (6) PCR amplified 102 bp product of ***sea*** gene Positive, Lane 1, 2, 3 and 4 are negative.

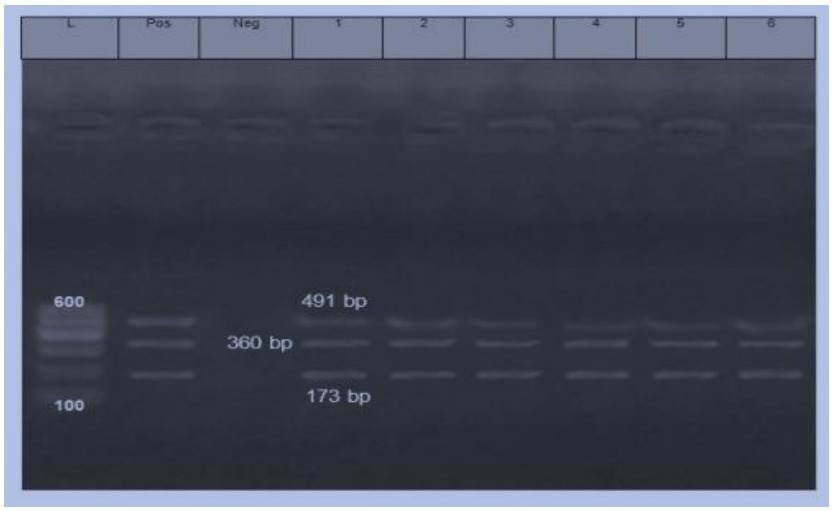

Figure (3): Agarose gel electrophoresis of products on a 2% agarose gel from multiplex PCR of (*blaZ, tetK, etb* and *aac* (6') *aph* (2") gene, Lane M: 100 bp DNA ladder, Lane (Pos.); positive control; Lane (N); negative controls; Lanes 1, 2, 3, 4, 5 and 6 PCR amplified 173 bp product of *blaZ* gene PCR amplified 360 bp product of *tet* gene, and PCR amplified 226 bp product of *etb* gene Lanes (6) PCR amplified 491 bp product of *aac*(6') *aph* (2")gene Positive, Lane 1, 2, 3 and 4 are negative.

Staphylococci clinical isolates will subjected to antimicrobial susceptibility testing. The genes implicated in resistance to peniillin (blaZ), gentamicin (aac(6')/aph(2"), and tetracyclin (tetK, tetM). Nizami *et al.* (2012).

CONCLUSION

Present study reports increasing prevalence of S. aureus isolates, the frequency of virulence genes and genetic resistance in the isolates is a main reason for treatment failure and possibly leads to spread of resistance in recurrent bovine mastitis in Egypt generally resistant to many of the antimicrobial compounds commonly used for treatment of mastitis, especially penicillin. Therefore Susceptibility testing and PCR are recommended as part of the diagnosis and essential tool for epidemiological studies. These help in selection of the most appropriate designing

strategic plans for therapeutic agents for treatment and control spread of S. aureus.

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الصفات الجزيئية لسموم المكور العنقودى الذهبى المقاوم لبعض المضادات الحيوية لالتهاب الضرع المتكرر فى الابقار الحلابة

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هذه الدراسه اجريت على عدد ١٧٩ ابقار حلابه من ثلاث مزارع ابقار تعاني من التهاب الضرع المتكرر. بالفحص البكتيريولوجى التقليدى لعينات الالبان من تلك الابقار الحلابه وجد انه لايوجد نمو بكتيرى فى عدد ١٥ عينه (٨,٤ %) ويوجد نمو بكتيرى فى عدد ١٦٤ عينه (٩١,٦ %) منهم عدد ٨٤ عينه (٤٦,٩%) تم عزل الميكروب العنقودى وعدد ٨٠ عينه (٤٤,٧%) ميكروبات اخرى وعن طريق التعريف الجزيئي بواسطة تفاعل البلمره المتسلسل وجد انه ٦٩ عتره من الميكروب العنقودى الذهبى (٣٨,٥%)، حيث تم تحديد النمط الظاهري والأنماط الجينية لبعض عوامل الضراوة انتيروتوكسين (A) و(B) على التوالى (*Sea* and Seb) بنسبة (١٠,١٪ و ١,٤٪) على التوالي وكذلك تحديد الجينات *blaZ* و aac(6')aph (2")) و *tetK* المقاومة للمضادات الحيويه البنسللين والجنتاميسين والتتراسيكلين بنسبة ١٠٠% و٤٠,٦% و٥٣,٦ % علي التوالي. أظهر ان تحديد النمط الجيني بواسطة تفاعل البلمره المتسلسل له دقة اكبر فى الكشف عن توصيف عوامل الضراوه والمقاومة لبعض المضادات الحيويه للميكروبات المستخدمة في علاج التهاب الضرع المتكرر للابقار الحلابه. تشير هذه الدراسة إلى وجود المكورات العنقودية الذهبية التى لها مقدره على فرز الانتيروتوكسين نوعى a , b وكذلك تحمل الصفات الخاصة المقاومة للمضادات الحيويه المتعددة المستخدمه فى علاج التهاب الضرع المتكرر للابقار الحلابه ومن الممكن أن تشكل عقبة رئيسية في علاج التهاب الضرع في مزارع الألبان وتؤدى الى خسائر لاقتصاديات المزرعه وكذلك امكانية انتقال مثل هذه الميكروبات الى الانسان.